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AWARD NUMBER: DAMD17-02-1-0668

TITLE: Development and Novel Uses of Antibodies in Epithelial Ovarian Cancer

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REPORT DATE: August 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-08-2004	Annual	15 Jul 03 – 14 Jul 04

Development and Novel Uses of Antibodies in Epithelial Ovarian Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0668

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

New York University School of Medicine
New York, New York 10016

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Immunologic approaches to treat ovarian cancer, a chemo-sensitive tumor, are in their infancy and have generally represented isolated clinical trial efforts. Further understanding of the host response to epithelial cancers and the potential capability of innovative immunologic technologies to ovarian cancer may play a key role in therapeutic advances. This integrated program proposes to expand the scope of ovarian cancer treatment in general, and immunotherapy in particular by working in three new and interrelated directions. These include: 1) new techniques that may identify relevant new human antibodies and characterize the target antigens, 2) test these in project models to evaluate the potential role in future therapy for ovarian cancer combined with radiation and chemotherapy, and 3) explore their role in allowing for the more accurate targeting of gene therapy. During the second year of the project, we were able to continue projects 1 - 3, culminating in the submission and acceptance of the abstract entitled The HUI77 Cryptic Epitope is Expressed in Human Ovarian Carcinoma and Regulates Tumor Cell Adhesion and Proliferation in vitro at the Society of Gynecologic Oncologists annual meeting.

15. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
Pharmacokinetics, Biodistribution, Immunoreactivity, Radiolabeling and Chelating monoclonal antibodies, Western Blot assays, Cell biology, Extracellular Matrix, Matrix, Metalloproteinases, Integrins

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	14	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction

Ovarian cancer is the leading cause of death due to gynecologic malignancies and is the fourth leading cause of death due to all cancers in women. The current challenges to reducing morbidity and mortality to this disease are well known. The majority of patients are diagnosed with advanced disease and, although the initial response to surgery and combination chemotherapy is high, the majority of patients will suffer a relapse. Second or third line therapies can often induce regression or disease stabilization, providing further evidence that ovarian cancer is a chemosensitive tumor. Therefore, we consider the identification of immune responses and antigens, immunomodulation of current active agents, and the introduction of novel treatment approaches by targeted gene therapy to hold the promise for further advances against ovarian cancer, and worthy of concerted multidisciplinary efforts by our clinical and basic scientists. Accordingly, the principal investigators have sought collaborations within the institution and at collaborating facilities to launch three interrelated preclinical therapeutics projects.

In project 1, investigators will utilize a fusion partner cell line that is capable of producing antibodies when fused with human B-lymphocytes derived from lymph nodes, or peripheral blood from patients with ovarian cancer. The production of human monoclonal antibodies will be characterized for their specificity to epithelial ovarian cancer and the type of antigens they recognize. Cell lines producing promising antibodies can then be further expanded and studied in the other preclinical projects designed to explore therapeutic models of combined modality and gene therapy. However, other potential applications, such as in diagnosis or vaccine development, could also be considered.

Project 2 utilizes monoclonal antibodies to target ovarian cancer cells testing monoclonal the potential therapeutic impact of antibody-linked radioisotopes combined with continuous exposure to topoisomerase 1 inhibitors. . Investigators in project 2 will build on past work with BrE-3 antibody and their long-standing interest in topoisomerase 1 as a target, the role of inhibitors as radiosensitizers, and the pharmacology of the clinically useful inhibitors. The model will also allow for rapid testing of new antibodies discovered in project 1.

The Laboratory shared resource would support these three studies by providing quality control, biodistribution and assessing the pharmacokinetics of the novel antibodies identified. The Administrative & Planning core will assist in the analysis of the results, and help plan experiments that may allow their transfer into clinical testing.

Thus, the studies proposed would integrate the identification of novel monoclonal antibodies with pre-clinical therapeutic potential as either direct anticancer agents, or as adjuncts to better targeting of either radioisotopes or gene therapy virus vectors. We anticipate that these studies would form the basis for exploring their potential in preclinical models of successful radioimmunotherapy and gene therapy transduction for therapeutic purposes, in situations that frequently arise during the course of ovarian cancer (e.g., presence of residual disease after initial chemotherapy, and of drug-resistant recurrences.)

Body

Core Facility B: Administration

Task Assignments and Status:

1. Establishment of executive committee to provide oversight of program project and communicate with internal and external participants
    i. Establishment of regular meeting cycles and review, report preparation

    Status: Continued in months 13 - 24

2. Establishment of internal advisory committee to provide feedback regarding program project

    Status: Continued in months 13 - 24

3. Establishment of external scientific advisory committee to provide oversight and scientific evaluation of program.

    Status: Continued in months 13 - 24

Core Facility A: Laboratory Core

Task Assignments and Status

1. Work in Support of Therapeutic Studies - Months 12 – 36
    Pharmacology Section: Support of Project 1 studies with therapeutic activity of ovarian specific antibodies in xenograft studies. Development of direct labeling or conjugation with bifunctional chelating agents for ^{188}Re labeling.

    Tissue and Immunopathology: Support project #1. Preparation of unstained paraffin block slides form ovarian cancer specimens to test specificity of monoclonal antibodies produced by hybridoma.

    Status: Continued in months 13 - 24

2. Work Extending the Project Scope with New Antibodies – Month 16 – 36
    Pharmacology Section: Conjugation of DOTA chelates with TIP-2 (or other human antibodies produced by Project 1) with the guidance of Dr. Michael Lewis. Assessment of immunoreactivity and stability with these new constructs. Preparation and calibration of osmotic infusion pumps.

Tissue and Immuopathology: Support of Projects 1-3. Assistance in the preparation of histological sections from murine ovarian xenograft models in the three projects. Analysis of intratumoral distribution of administered antibodies and targeted antigens.

Status: Continued in months 13 - 24

Project 1: Development and Antigen Characterization of Totally Human Monoclonal Antibodies to Ovarian Cancer-Associated Antigens

Task Assignments and Status:

1. To develop a panel of hybidomas using ovarian cancer patients' retroperitoneal or pelvic lymph nodes and peripheral blood lymphocytes (months 1 – 18)
    a. Collect clinical material (peripheral blood and lymph nodes) to isolate lymphocytes for hybridization with human hybridoma fusion partner cells MFP-2.
    b. Screen hybridoma panels for the secretion of ovarian cancer cell specific monoclonal antibodies (months 4 – 18)
2. Establish fusion partner cell lines from human B-lymphocytes derived from ovarian cancer patients.
3. Identify candidate antibodies capable of targeting established human ovarian cancer cell lines

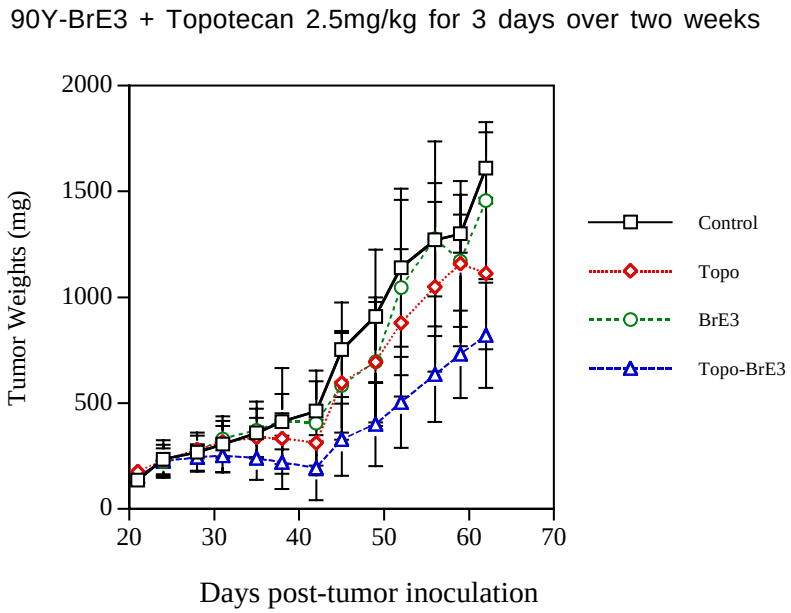
Status

- In collaboration with the laboratory core and the Tissue procurement service core of the NYU Cancer Center lymph node samples from patient with ovarian cancer were obtained at NYU Medical Center, processed and transferred to Columbia University. A total of 30 patients were consented and had lymph nodes processed in 2004.
- Fusion partner cell lines were derived from these lymph nodes. Initial studies have identified unique monoclonal antibodies being produced by these cell lines. Characterization of these antibodies has begun.
- TIP-2, a monoclonal antibody identified in a previous experiment was studied to determine if there was immunoreactivity against ovarian cancer.

Project 2: Potentiation of radioimmunotherapy by topoisomerase I inhibitor when combined for ovarian cancer treatment.

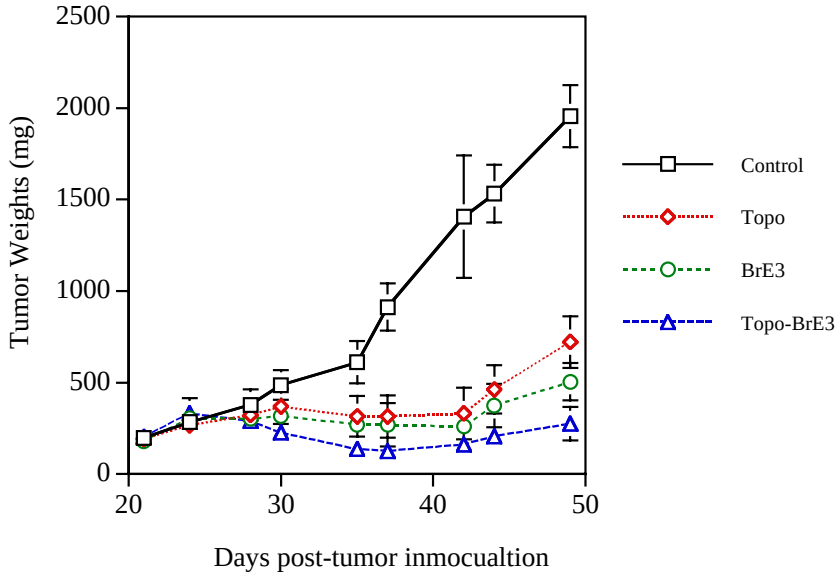
Objective 1: Demonstrate the efficacy of huBrE-3/Topotecan in ovarian cancer *in-vivo* models and extend this model to a strategy employing : huBrE-3 /Topotecan CI or Topotecan I.P.

We have completed tumor response experiments comparing three different schedules of topotecan when combined with Y-90 huBrE-3 at 200 uCi: continuous infusion at 14 mg/kg over two weeks (using subcutaneously implanted Alzet diffusion pumps), single weekly intraperitoneal injections of 7 mg/kg for two weeks, and three consecutive daily injections each week for two weeks. Results were presented at the AACR-EORTC meeting in Boston, November, 2003. (abstract appended). These results show that on the three daily injections each week for two weeks, only combined treatment significantly reduced tumor size relative to controls (Kruskal Wallis test p = 0.0101).



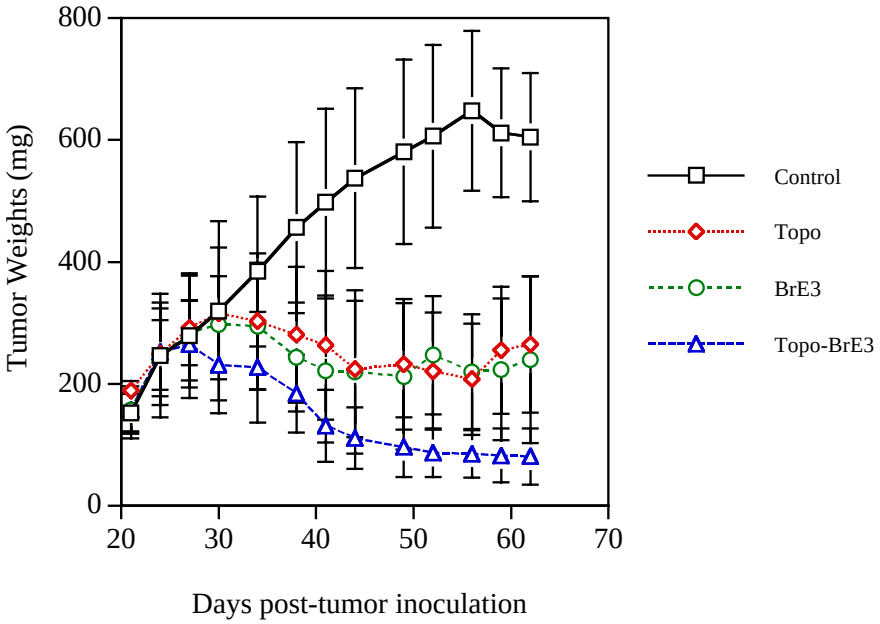
For the continuous infusion schedule, there was significant tumor inhibition by Topotecan on this schedule combined with Y-90 huBrE-3 radioimmunotherapy compared to either single agent or untreated control in terms of mean tumor weight. (Day 49 – Day21) among the four treatment groups (KW; $p<0.0001$). After adjusting for multiplicity ($p<0.01$) significant differences were seen between all treatments and control (Topotecan+ radioimmunotherapy ($p=0.0034$), Topotecan alone ($p=0.0058$), radioimmunotherapy ($p=0.0034$)). There was also a significant difference between the cohort treated with the combination compared to either radioimmunotherapy alone ($p=0.0036$) or Topotecan alone ($p=0.001$).

90Y-BrE3 + 14 days continuous s.c. infusion of Topotecan (1mg/kg/day)



For the two weekly single intraperitoneal injections of topotecan, four group comparison of mean tumor weight change over this interval (Day 62-Day 21) showed that single agent and combination treatment yielded significant tumor responses relative to controls (KW; p = 0.0004).

90Y-BrE3 +Topotecan 7mg/kg i.p. two weekly treatment



Adjusting for five multiple comparisons, each treatment group showed reduced average tumor weight compared to untreated controls (Topotecan plus radioimmunotherapy (p=0.0024), Topotecan alone (p=0.0024), radioimmunotherapy (p=0.0037)). The combination regimen had a greater reduction than Topotecan alone (p=0.0027), but not

radioimmunotherapy alone. These results suggest that the continuous infusion may have an advantage over the other schedules when used in combination. In a clinical setting where patients may not be as tolerant of dose intensity of either agent when used in combination, the superiority of the continuous infusion topotecan combined with Y-90 huBrE-3 over single agent, may offer the most synergy.

In addition we have tested several doses of cisplatinum administered once weekly either one time or twice and have now determined the MTD of cisplatinum in combination with this regimen to be 3mg/kg once a week for two weeks. Using this dose level, we then performed a tumor response experiment comparing untreated controls with four treatment groups: Y-90 Bre3 alone, continuous infusion topotecan plus cisplatin, topotecan plus Y-90 hBre3 and continuous infusion topotecan plus cisplatin plus Y-90 huBre3.

The following table shows the mean tumor weight (mg), its standard deviation and number of mice in each group.

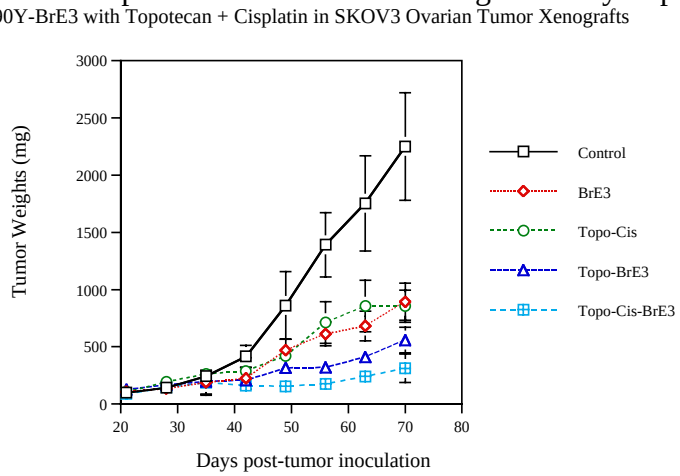
#	Group	Day 21	Day 70	Change in Weight (Day 70 – Day 21)
0	**Control (n = 4)**	98.7 ± 31.2	2250.1 ± 469.8	2151.4 ± 489.3
1	**Bre3 (n = 8)**	106 ± 53.6	893.9 ± 163.2	787.9 ± 158.5
2	**T + C (n = 8)**	105.8 ± 53.1	855.4 ± 139.9	749.6 ± 112.4
3	**T + C + Bre3 (n = 8)**	88.5 ± 22.3	312.7 ± 124.9	224.3 ± 127.9
4	**T + Bre3 (n = 6)**	126.8 ± 54.5	558.8 ± 111.5	431.9 ± 132.7

An assessment of the differences in weight among the 5 groups at day 21 indicated that there are no statistically significant differences among the groups (Kruskal-Wallis chi-square = 1.64, df = 4, p = 0.8).

The Kruskal-Wallis test was used to examine changes in tumor weights over the period from 21 to 70 days among the 5 treatment groups. It tests the null hypothesis that there is no difference in the change in tumor weight (Day 70 – Day 21) among the five groups against an alternative hypothesis of differences among the groups. A statistically significant difference was observed among the five groups (Chi-square = 27.4, df = 4, p-value < 0.0001).

Further pair-wise comparisons of interest were made using the Wilcoxon rank sum test. Significantly smaller increases in tumor weight were observed over the 49 day period in animals in all treatment groups compared with control animals (Bre3 (p = 0.0085), topotecan + cisplatin (p = 0.0085), topotecan + cisplatin + Bre3 (p = 0.0085) and topotecan + bre3 (p = 0.014)) and the combination of topotecan + cisplatin + bre3 and topotecan + bre3 (p = 0.02). No adjustments are made for multiplicity. This data

suggests that the addition of cisplatin to the combination regimen may improve



therapeutic efficacy.

Additionally, we have begun to test other agents in the topotecan/Y-90huBrE3 combination. In vitro studies assessing the effect of bortezomib (0.1 uM), a proteasome inhibitor have shown a significant increase in growth inhibition when added to topotecan alone that is equivalent to combining radioimmunotherapy and topotecan at 0.1 and 1.0 uM concentrations of topotecan. At higher concentrations of topotecan, the combination of all three agents yielded a much greater growth inhibition. Based on observations made by Dr. Brooks in melanoma cells, we have tested the effects on SKOV3 cells of this regimen of prior exposure (for 7 days) to para-aminobenzoic acid. This has shown an increase in growth inhibition by the addition of PABA to either topotecan as compared to drug alone, by adding PABA to radioimmunotherapy combined with topotecan and wotj slightly greater (but not significantly so) inhibitory effect by using all three in combination (with an increase in growth inhibition by PABA plus radioimmunotherapy plus topotecan compared to topotecan plus radioimmunotherapy. Furthermore, while the effect of adding PABA appears to be more dramatic in cells only exposed to topotecan for 24 hours, there is a trend to an overall greater effect when cells are exposed for 48 hours. We also investigated the combination of all four agents in vitro. The combination of PABA for 7 days, topotecan at 0.1 uM, Y-90 huBrE-3 (1 uCi) and bortezomib (0.1 uM) showed significantly greater growth inhibition at 48 hours after treatment compared to topotecan alone, Y-90 huBrE-3 alone, topotecan combined with bortezomib, or Y-90 huBrE-3 combined with topotecan alone.

Objective 2: Identify the most effective antibody in the combinations: huBrE-3/ chemotherapy vs TIP-2 (or other human antibodies produced by Project 1)/ chemotherapy

One very frustrating aspect has been our inability to obtain the required amounts of antibody from Project 1 as an alternative antibody. The originally proposed antibody TIP2 has been shown to recognize an intracellular epitope suggesting that the anibody will not bind very well in vivo. Another antibody identified by Dr Trakht does recognize a cell surface epitope, but scale up to provide sufficient amounts has not been achieved. The possibility of an alternative antibody (from project 3) is currently being investigated.This antibody recognizes a cryptic epitope of denatured Collagen Type IV that is present in tumor vascular basement membrane and intercellular tumor matrix. The

potential to compare this with huBrE-3, which recognizes a cell surface antigen, as a vector of radioimmunotherapy offers exciting possibilities. A response from the producer of this antibody (CancerVax) is expected in the next couple of months.

Objective 3:

Define the optimal isotope for radioimmunotherapy in this system: ^{90}Y vs ^{188}Re

We have now successfully conjugated BrE-3 with MAG-3 for labeling with either Tc-99m and are now testing cold perrhenate with this complex. Initial attempts to label with HYNIC markedly diminished the immunoreactivity of the antibody probably due to breakdown of the IgG itself as indicated by HPLC. We then proceeded to conjugate the antibody with MAG-3. Radiolabeling with Tc-99m showed high efficiency, maintenance of the integrity of the antibody, and preservation of the immunoreactivity. Because of the expense of the W-188/Re-188 generator, our strategy has been to use mass spectroscopy to test the ability of the BrE-3-MAG3 to bind perrhenate. We have now purified the MAG-3-BrE-3 complex and determined our ability to measure and detect the integrity of that molecule using mass spectroscopy. Our next step is to evaluate Re-MAG-3-BrE-3 using mass spectroscopy. Once we have shown that we can use mass spectroscopy to assess the "labeling", we will then be able to proceed with immunoreactivity and stability measurements. Should MAG-3 perform well with Rhenium, we will be able to proceed with Re-188 for in vitro and in vivo testing in comparison to Y-90 huBrE-3.

Objective 4:

Mechanistic approaches for the combined activity

We have also started to investigate the interaction of treatment with Y-90 huBrE-3 and topotecan treatment in vitro, examining cell cycle effects of radioimmunotherapy to test the hypothesis that S-phase synchronization occurs with low dose radiation exposure delivered by this radioimmunoconjugate. These results have been inconclusive and require repeating.

We have evaluated the differences in tumor, blood and normal organ pharmacokinetics of Y-90 hu BrE-3 in mice treated with topotecan compared to mice treated with radioimmunotherapy alone. The experiment was performed in two phases. The first experiment was interrupted by the NYC blackout last August and was therefore only carried out to 72 hours, but the second set of animals were studied through 7 days after administration of radioimmunoconjugate. Both of these studies show an increased percent injected dose of Y-90 huBrE3 per gram in tumor for the combination treatment compared to Y-90-HuBrE3 alone. There is also a trend that suggests that the area under the curve for blood levels of Y-90 huBrE-3 was smaller in animals treated with the combination. Two explanations for the trend in changes in blood pharmacokinetic occur: one is that topotecan is affecting other organs and the metabolism of the antibody, but since no significant differences are seen for the pharmacokinetics of normal organs, it is more likely that the relative size of the tumor in the mouse and the change of tumor uptake actually increases the clearance from the blood of the radioimmunoconjugate. While the increased tumor uptake after administration of topotecan is expected to occur in patients, the relative tumor burden in patients is not likely to be so large that changes in blood pharmacokinetics will be seen. These results were presented at the Society for Gynecologic Oncology in San Diego, CA in February, 2004.

Project 3 Targeting Cryptic Epitopes as a Novel Therapeutic Strategy for Treatment of Ovarian Carcinoma

Task Assignments and Status:

1. Months 13-24 To examine the cellular and biochemical consequences of collagen proteolysis on ovarian carcinoma cell adhesion, migration, invasion, proliferation, and cell survival.

Status

1). Effects of Mab HUI77 Ovarian Carcinoma Cell Adhesion in Vitro.
Our previous studies have indicated that the HU177 cryptic collagen epitope was specifically exposed within the ECM of human ovarian carcinomas but not in normal ovarian tissues. To examine whether ovarian tumor cells utilize the HU177 cryptic epitope within collagen to regulate adhesion, in vitro adhesion assays were carried out. Optimal working conditions were established for the in vitro adhesion assays for both collagen type-I and collagen type-IV. As shown in figure 1A and B, SKOV-3 human ovarian carcinoma cells attach to denatured collagen type-I (A) and denatured collagen type-IV (B). Interestingly, Mab HU177 dose dependently inhibited SKOV-3 cell adhesion to denatured collagen type-I and IV, with maximum inhibitory activity observed at a concentration of 200ug/ml. Mab HU177 had little if any effects on SKOV-3 cell adhesion on intact collagen type-I or IV. In further control experiments, Mab HU177 had no effect o f carcinoma cell adhesion to other ECM proteins such as fibronectin in either its native or denatured forms. Additional adhesion studies were carried out with Mab XL313 directed to a cryptic epitope within collagen type-I. While some limited inhibitory activity was observed further optimization and dose studies will be necessary to draw specific conclusions. Taken together these studies provide evidence that the HU177 cryptic epitope may play a functional role in regulating human ovarian carcinoma cell adhesion to structurally modified collagen types-I and IV. Additional studies are underway to establish the working conditions for maximum exposure of the cryptic collagen epitope recognized by Mab XL313.

2). Effects of Mab HUI77 Ovarian Carcinoma Cell Migration in Vitro.
Our current studies suggest that the HU177 cryptic collagen epitope may play a functional role in regulating ovarian carcinoma cell adhesion. Given the role of tumor cell adhesion to ECM components in the regulation of motility, we examined the effects of Mab HU177 on SKOV-3 cell migration. Optimal working conditions were established for the in vitro migration assays for both collagen type-I and collagen type-IV. As shown in figure 2A and B, SKOV-3 human ovarian carcinoma cells migrated on denatured collagen type-I (A) and denatured collagen type-IV (B). Interestingly, Mab HU177 inhibited SKOV-3 cell migration on denatured collagen type-I and IV by approximately 60 to 70% as compared to controls with maximum inhibitory activity observed at a concentration of 200ug/ml. Mab HU177 had little if any effects on SKOV-3 cell migration on intact collagen type-I or IV. In further control experiments, Mab HU177 had no effect on carcinoma cell migration on other ECM proteins such as

fibronectin in either its native or denatured forms. Taken together these studies provide evidence that the HU177 cryptic epitope may play a functional role in regulating human ovarian carcinoma cell migration on structurally modified collagen types-I and IV.

3). Establishment of the Optimal Conditions for SKOV-3 Ovarian Carcinoma Cell Proliferation. We have now established the experimental conditions to assess the effects of Mab HUI77 on ovarian carcinoma cell proliferation in vitro on both denatured collagen type-I and denatured collagen type-IV. In particular, coating concentrations of collagen were established as well as an optimal timer course and antibody concentrations were also determined. Proliferation will be monitored by measuring the tumor cell associated mitochondrial dehydrogenase with a commercially available assay kit (WST-Cleavage). Our preliminary results indicate that Mab HUI77 (200ug/ml) potently inhibited (approximately 60%) SKOV-3 cell proliferation on denatured collagen type-I. Additional experiments are underway to assess the effects of the Mab HU177 on proliferation of SKOV-3 cell on Denatured collagen type-IV. Interestingly, our preliminary studies also suggest that the inhibitory activity observed with Mab HU177 on SKOV-3 cells may involve regulation of the expression of cyclin dependent kinase inhibitors. In this regard, we have now established the working condition to determine the effects of Mab HU177 on the relative levels of $P21^{CIP1}$ mRNA by real time RT-PCR.

4). Establishment of Optimal Condition for the In Vivo Chick Embryo Tumor Growth Assay. To begin to establish the optimal experimental conditions to examine the effects of Mab HU177 on ovarian tumor growth we used the chick embryo tumor growth assay. Briefly, subconfluent cultures of human ovarian carcinoma cell line SKOV-3 were harvested, washed and resuspended in sterile PBS. The CAMs of 10-day old chick embryos were prepared and SKOV-3 cells were added to the CAMs. Twenty-four hours later the embryos were injected systemically with a single injection of Mab HU177 or an iso-type-matched control antibody. Studies indicated that a 100ul was the maximum volume tolerated by the chick embryos. Thus all antibody concentrations were adjusted to a maximum of 100ul per injection.

Future Plans:

We have made significant progress towards the major objectives of our proposal.

1). We plan to continue our assessment of the effects of both Mabs HUI77 and XL313 on SKOV-3 carcinoma cell proliferation and regulation of gene expression. In addition, we plan to establish the working conditions needed for optimal exposure of the XL313 cryptic epitope within collagen type-I.

2). Finally we plan to further study the effects of both Mab HU177 and XL313 on SKOV-3 tumor growth in vivo.

Key Research Accomplishments

- Preliminary results indicate that mab HU177 inhibits growth of SKOV-3 cells grown on denatured collagen matrix
- Established chick embryo/ovarian cancer model which will allow for further study of effects of mabHU177

Reportable Outcomes

Title: The HUI77 Cryptic Epitope is Expressed in Human Ovarian Carcinoma and Regulates Tumor Cell Adhesion and Proliferation in vitro

Authors: Nimesh P.Nagarsheth, Anat Zelmanovich, Jennifer M. Roth, John P. Curtin, Peter C. Brooks

Objectives: Cryptic epitopes of the extracellular matrix (ECM) have been shown to regulate tumor growth. HU177 (provided by CMI, a subsidiary of CancerVax Corp.) is a monoclonal antibody (Mab) that reacts with denatured collagen, but exhibits minimal reactivity with triple helical collagen (THC). We examined the possibility that the HUI77 epitope was exposed in human ovarian cancer and tested whether cellular interaction with this epitope regulates ovarian cancer behavior.

Methods: Solid phase ELISAs were used to examine the reactivity of Mab HU177 with types I and IV denatured and THC. In vitro adhesion and proliferation assays were used to assess the effect of Mab HUI77 and anti-integrin antibodies on SKOV3 human ovarian carcinoma cell adhesion and proliferation on types I and IV denatured and THC. Immunohistochemistry was performed with Mab HU177 on malignant and normal ovarian tissue. Statistical analysis was performed using the Kruskal-Wallis test.

Results: Mab HU177 specifically reacts with denatured collagen-I and IV while exhibiting minimal reactivity with THC. Mab P4C10 (anti-b1 integrin) completely blocked SKOV3 cell adhesion to THC type-I and IV, while Mabs LM609 (anti-avb3 integrin) and P1F6 (anti-avb5 integrin) had no effect. In contrast, SKOV3 cell adhesion to denatured collagen type-I was partially inhibited by Mabs LM609 and P4C10. Mab HU177 significantly inhibited (up to 80%) adhesion of SKOV3 cells to denatured collagen type-I ($p=0.0001$) and IV ($p=0.001$). Mab HU177 significantly inhibited SKOV3 cell proliferation at 72 hours on both denatured collagen-I ($p=0.001$) and IV ($p=0.0031$). The HUI77 epitope was highly expressed within the ECM of human ovarian carcinomas, while little if any was detected within normal ovarian tissue.

Conclusions: Human SKOV3 ovarian carcinoma cells can interact with denatured collagen and this interaction was mediated in part by b1 and avb3 integrin receptors. Interestingly, Mab HUI77 significantly inhibited SKOV3 cell adhesion and proliferation on denatured collagen. These findings, in conjunction with the expression of the HUI77 epitope in human ovarian tumors, suggest that the HUI77 epitope may play an important role in regulating ovarian tumor growth. Currently, studies are underway to determine whether the HUI77 cryptic epitope represents a useful target for treatment of ovarian carcinoma in vivo.